

09/29/98  
JCS 598 U.S. PTO

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09/163289



September 29, 1998

Attorney Docket No.: 07265/098002

## Box Patent Application

Assistant Commissioner for Patents  
Washington, DC 20231

Presented for filing is a new divisional patent application of:

BOSTON  
HOUSTON  
NEW YORK  
  
SOUTHERN CALIFORNIA  
  
SILICON VALLEY  
TWIN CITIES  
  
WASHINGTON, D.C.

Applicant: HARRY C. DIETZ  
Title: DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS AND METHODS OF USE

Enclosed are the following papers, including all those required to receive a filing date under 37 CFR §1.53(b):

	<u>Pages</u>
Specification	29
Claims	2
Abstract	1
Declaration	1
Drawing(s)	4

### Enclosures:

- Small entity statement (1 page). This application is entitled to small entity status.
- Postcard.
- Permission to use Sequence Listing, 4 pages.
- Information Disclosure Statement, 1 page.
- Amendment filed 8/18/97, 16 pages.
- Amendment filed 1/20/98, 5 pages.

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Date of Deposit September 29, 1998

I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Duncan Clark

DUNCAN CLARK

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BOX PATENT APPLICATION

September 29, 1998

Page 2

This application is a divisional (and claims the benefit of priority under 35 USC §120) of U.S. application serial no. 08/742,943, filed October 31, 1996 . The disclosure of the prior application is considered part of (and is incorporated by reference in) the disclosure of this application.

Preliminarily, on page 1 of the specification, before line 1, insert --This is a divisional of U.S. application serial no. 08/742,943, filed 10-31-96, (pending).--

Basic filing fee	\$ 395.00
Total claims in excess of 20 times \$11.00	0.00
Independent claims in excess of 3 times \$41.00	0.00
Multiple dependent claims	0.00
Total filing fee:	\$ 395.00

A check for the filing fee is enclosed. Please apply any other required fees or any credits to deposit account 06-1050, referencing the attorney docket number shown above.

If this application is found to be INCOMPLETE, or if a telephone conference would otherwise be helpful, please call the undersigned at 619/678-5070.

Kindly acknowledge receipt of this application by returning the enclosed postcard.

Please send all correspondence to:

Lisa A. Haile  
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Respectfully submitted,



Lisa A. Haile, Ph.D.  
Reg. No. 38,347

Enclosures

70193.LJ1

Applicant or Patentee: Harry C. Dietz  
 Serial or Patent No.:  
 Filed or Issued: October 31, 1996  
 For: DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS AND METHODS OF USE

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
 (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: The Johns Hopkins University School of Medicine  
 Address of Organization: 720 Rutland Avenue, Baltimore, Maryland 21205  
 Type of Organization:

- UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION  
 TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))  
 NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA  
 (NAME OF STATE: )  
 (CITATION OF STATUTE: )  
 WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA  
 WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA  
 (NAME OF STATE: )  
 (CITATION OF STATUTE: )

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS AND METHODS OF USE by inventor(s) Harry C. Dietz described in

- the specification filed herewith.  
 application serial no. \_\_\_\_\_, filed October 31, 1996.  
 patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name: \_\_\_\_\_

Address: \_\_\_\_\_

INDIVIDUAL     SMALL BUSINESS CONCERN     NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: Howard W. Califano

Title: Assistant Dean and Director

Address: 720 Rutland Avenue, Baltimore, Maryland 21205

Signature: Howard W. Califano

Date: 11-19-96

PATENT  
ATTORNEY DOCKET NO. 07265/098001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Harry C. Dietz                          Art Unit: 1804  
Serial No.: 08/742,943                          Examiner: D. Clark  
Filed : 10/31/96  
Title : DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS AND  
METHODS OF USE

Assistant Commissioner for Patents  
Washington, DC 20231

AMENDMENT

In response to the Office Action dated May 16, 1997, in connection with the above-identified application, please consider the following amendments and remarks:

Date of Deposit August 18, 1997

I hereby certify under 37 CFR 1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Lucille M. Begalla

Lucille M. Begalla

In the claims:

Please amend the claims as follows:

1. (Amended) A nucleic acid construct for suppressing expression of a target gene [expression], comprising:

an antisense nucleic acid sequence directed to a target gene of interest;

an unmodified, naturally occurring 5' U snRNA 5' of said antisense nucleic acid sequence stem loop structure;

[an antisense nucleic acid; and]

a pol II promoter region 5' of said antisense nucleic acid sequence ; and

3' of said antisense region an unmodified, naturally occurring 3' U snRNA stem loop structure [.], wherein said expression of said target gene is suppressed by at least 75% of the normal level of expression.

Please cancel claim 2.

2. *The nucleic acid construct of claim 1, wherein the stem loop structures are unmodified U snRNA structures.*

3. (Amended) The nucleic acid construct of claim [2] 1, wherein the U snRNA is U1.

Please cancel claim 4.

4. *The nucleic acid construct of claim 1, further comprising a promoter.*

5. (Amended) The nucleic acid construct of claim [4] 1, wherein the promoter is a U1 snRNA promoter.

6. (Amended) The nucleic acid construct of claim [4] 1, wherein the promoter is a constitutive promoter.

7. (Amended) The nucleic acid construct of claim [4] 1, wherein the promoter is an inducible promoter.

8. (Reiterated) The nucleic acid construct of claim 1, further comprising a ribozyme nucleic acid.

9. (Reiterated) The nucleic acid construct of claim 8, wherein the ribozyme nucleic acid is located between the 5' and 3' loop structures.

10. (Reiterated) The nucleic acid construct of claim 8, wherein the ribozyme nucleic acid is a hammerhead-type ribozyme.

11. (Reiterated) The nucleic acid construct of claim 8, wherein a consensus sequence for ribozyme cleavage in a target nucleic acid is 5'-GUC-3' or 5'-GUA-3'.

12. (Amended) The nucleic acid construct of claim 1, wherein the antisense nucleic acid is targeted to a region of a gene is selected from the group consisting of rent-1, HPV E6, HIV, hyaluronic acid synthase, and fibrillin.

13. (Amended) A method for suppression of gene expression comprising administering to a cell a suppressive-effective amount of the nucleic acid construct of claim 1[, whereby expression of the gene is suppressed].

Please cancel claim 14.

14. *The method of claim 13, wherein the administering is in vivo.*

15. (Reiterated) The method of claim 13, wherein the administering is *in vitro*.

Please cancel claim 16.

16. *The method of claim 13, wherein the administering is ex vivo.*

17. (Amended) The method of claim 13, further comprising:

administering a [modified] second nucleic acid encoding a wild-type polypeptide

corresponding to the gene product of the gene being suppressed, wherein the [modified] second nucleic acid is resistant to ribozyme cleavage and/or antisense inhibition.

Please cancel claims 18 and 19.

18. *A transgenic animal having the nucleic acid construct of claim 1.*

19. *A knock-out animal produced utilizing the nucleic acid construct of claim 1.*

Please add new claim 20:

--20. The nucleic acid construct of claim 1, further comprising a 5' trimethylguanosine cap.--

#### REMARKS

Claims 1, 3, 5-13, 15, 17, and 20 are pending. Claims 2, 4, 14, 18, and 19 have been canceled without prejudice. Applicant retains the right to pursue these claims in a later filed divisional or continuation application. Claims 1, 3, 5-7, 12, 13, and 17 have been amended. Claim 1 has been amended to incorporate the limitations of claim 2 and recite the use of "naturally occurring" stem loop structures. Support for this language can be found on page 7, line 12-14. Claim 1 has been amended to set forth the degree of gene suppression. Support for the amending language can be found on page 15, line 8. Claim 1 has also been limited by the inclusion of an "operatively associated pol II regulatory nucleotide sequence." Support for the amending language can be found on page 11, lines 22 to page 13, line 2). Claims 3, 5, 6, and 7 have been amended to change their dependency from a canceled claim. Claim 12 has been

amended to more specifically delineate the claimed invention. Claim 13 has been amended to more distinctly claim the subject invention. Claim 17 has been amended to remove the term "modified." Claim 20 has been added. Support for new claim 20 can be found on page 15, lines 1-2. Applicant notes the Examiner's request to comply with the sequence rules under 37 CFR 1.821-1.825 and will file a completed sequence listing within the statutory period.

#### Summary of the invention

The subject invention relates to a novel nucleic acid construct for delivery of antisense sequences for suppressing target gene expression. The construct includes a pol II promoter region, a 5' and a 3' unmodified U snRNA stem loop structure, and an antisense region located between the 5' and the 3' stem loop structures. Optionally, a ribozyme and/or a 5' trimethylguanosine cap are included in the construct.

#### Restriction Requirement

Applicants affirm the provisional election of the claims of Group I (claims 1-13, 15, and 17) drawn to a nucleic acid construct) with traverse and cancel claims 14, 18, and 19 without prejudice to renewal. Claim 14 has been placed in Group II, drawn to a nucleic acid construct and method of introducing said construct in vivo. Group II also includes Claims 1-13 and 17, which can also be classified in Group I (the elected invention). Claim 16 has been placed in Group III, which also includes claims 1-13 and 17). Applicants respectfully submit that if Claim 14 can be grouped with Claims 1-13 and 17, and Claim 16 can be grouped with Claims 1-13 and 17, the search required for the subject matter of Group I (Claims 1-13, 15, and 17) must include the subject matter of Group II(Claims 1-14, and 17) and Group III (Claims 1-

13, 16, and 17). Thus, examining the claims of groups I, II, and III would pose no undue burden upon the Examiner.

In view of these remarks, the Examiner is thus respectfully requested to withdraw the restriction requirement and examine Claims 1-17 in the present application.

Rejections Under 35 U.S.C. §112, first paragraph

Claims 1-13, 15, and 17 are rejected for allegedly not delineating the relationship between the 5' and 3' stem loops and the antisense nucleic acid. Applicant respectfully traverses this rejection.

The Office Action states that the specification is enabling for "a nucleic acid construct comprising 5' and 3' U snRNA stem loop structures and a nucleic acid which is antisense to a gene of interest, wherein the antisense nucleic acid is between said 5' and 3' stem loops." Claims 2 and 4 have been canceled, and the amendments to claims 1,3, 5-7, 12, 13, and 17 to more distinctly point out the relationship of the 5' and 3' stem loops and the antisense nucleic acid, are believed to render this rejection moot.

Claims 1-13, 15, and 17 stand rejected for the breadth of the term "antisense nucleic acid," which the Office Action states could be directed to something not within the gene of interest. Applicant respectfully traverses this rejection.

Claims 2 and 4 have been canceled. Claims 1 has now been amended to recite "an antisense nucleic acid sequence directed against a target of interest." The Office action states that "the breadth of the claim is non-enabling with respect to the anti-sense nucleic acid." The pessimistic statements about antisense nucleic acids presented in the Office Action are not an indication that antisense will not work. The potential of antisense oligonucleotides to

selectively inhibit protein synthesis from a target gene of interest has generated a great deal of enthusiasm for their development of experimental therapeutics (e.g. McShan, W.M., et al. (1992) *J. Biol. Chem.* 267:5712-21; Calbretta, B. (1991) *Cancer Research* 51:4505-4510), and the production of antisense oligonucleotides has further been a subject of patents (e.g. Noonberg et al., U.S. Patent 5,624,803.)

The specification provides ample guidance for production of antisense oligonucleotides and triple helix oligonucleotides. Furthermore the specification recites methods for introducing the antisense molecules into cells (page 13, lines 3 to page 14, line 7).

Applicants submit that the introduction of nucleic acids into cells (across the membrane) is routine to one skilled in the art, and that the methods of calcium phosphate, liposome /DNA complexes, and ligand/DNA conjugates and not limited to specific cell types (for review see Cooper, M.J., 1996, "Noninfectious Gene Transfer and Expression Systems for Cancer Gene Therapy," *Seminars in Oncology* 23(1):172-87).

In addition, a number of methods are known in the art for determining whether a particular antisense reagent will inhibit gene expression. Screening of candidate oligonucleotides by any one of these methods requires no more than routine screening, and is not undue experimentation. The following articles demonstrate the level of skill in the art for selection of appropriate antisense reagents. Duroux *et al.* (1995) N.A.R. 23:3411-3418, entitled "Rational design of point mutation-selective antisense DNA targeted to codon 12 of Ha-ras mRNA in human cells". Hyndman *et al.* (1996) Biotechniques 20:1090-1094, entitled "Software to determine optimal oligonucleotide sequences based on hybridization simulation data". DeDionisio and Lloyd (1996) J. Chromatogr. A 735:191-208, entitled "Capillary gel electrophoresis and antisense therapeutics. Analysis of DNA analogs". Ho *et al.* (1996) N.A.R.

24:1901-1907, entitled “*Potent antisense oligonucleotides to the human multidrug resistance-1 mRNA are rationally selected by mapping RNA-accessible sites with oligonucleotide libraries*”.

Using the art-established methods for selection of oligonucleotides and the direction provided by the subject application, a person of ordinary skill in the art could practice the claimed invention without undue experimentation. Withdrawal of the rejection is requested.

The Examiner rejects claims 8-11, citing a references indicating various difficulties in the practice of ribozyme techniques. However, difficulties alone do not render an application non-enabling: enablement does not require a guarantee of success or an absence of obstacles. All therapies encounter problems in development. Applicants note that a number of antisense/ribozyme compositions are currently in clinical trials, including treatments for CMV, genital warts, kidney transplant rejection, autoimmune diseases such as rheumatoid arthritis, chronic myelogenous leukemia, and AIDS. An antisense treatment for AIDS is currently in Phase IB/II trials (Hybridon GEM® 91) and an antisense treatment for CMV is currently in Phase I/II trials (Hybridon GEM® 132). Applicants respectfully request withdrawal of the rejection.

Claim 12 is rejected for reciting specific antisense nucleic acids. The Office Action states that no direction or guidance is presented to enable one of skill in the art to make the construct for some of the genes. Applicants respectfully traverse. A construct of the invention which targets RENT1 is described in the specification on page 14, lines 16-19, and is shown in Figure 1). A construct which targets fibrillin expression is described in the specification on pages 23, line 7 to page 25, line 20, and the effectiveness of this construct is further described on page 25 line 21 to page 27, line 25. Examples of other useful constructs are described in the specification on page 9, line 7 to page 10, line 2. Given the number of methods

well-known in the art for screening oligonucleotides (see above), applicants submit that ample guidance is available to make any construct, especially RENT-1, HPV E6, HIV, hyaluronic acid synthase, and fibrillin, which are expressly disclosed in the specification. Withdrawal of the rejection is requested.

Rejection Under 35 U.S.C. 112, second paragraph

Claim 1 is rejected for not reciting a specific range of suppression. While Applicant respectfully traverses this rejection, Claim 1 has now been amended to recite "wherein said expression of said target gene is suppressed by at least 75% of the normal level of expression" rendering this rejection moot.

Claims 2 and 3 are rejected for the use of the term "unmodified." Claim 2 has been canceled. However, the limitations of claim 2 have been incorporated into claim 1, thus this rejection will be addressed as it applies to amended claim 1 and to dependent claim 3.

Applicants respectfully traverse, and submit that the term "unmodified" is adequately defined in the specification on page 7, lines 17-23, as follows:

"The term "unmodified" means that the folding pattern of the stem loop structure is not compromised by alterations in the nucleic acid sequence of the naturally occurring molecule."

Examples of what would constitute an "unmodified" stem loop are also given on page 7.

Applicants would like to remind the examiner of MPEP section 608.01(o), which states:

"A term used in the claims may be given a special meaning in the description. No term may be given a meaning repugnant to the usual meaning of the term."

Applicants submit that the term "unmodified" is described in the specification in accordance with

MPEP 608.01(o), and thus removal of the rejection is respectfully requested.

Claim 12 has been rejected for being indefinite in its recitation of "...wherein the antisense nucleic acid is selected from the group consisting of..." Claim 12 has been amended to more distinctly point out the Applicant's invention, rendering this rejection moot.

Claim 13 is rejected for its use of the term "suppressive-effective amount." Applicants submit that the term is defined on page 15, lines 4-13, and that the use of the term "suppressive-effective" is in accordance with MPEP 608.01(o) (see above.) Thus Applicants respectfully request withdrawal of the rejection.

Claim 13 is rejected for the limitation "the gene" in line 3. This rejection is rendered moot by the amendments to claim 13.

Claim 17 is rejected for being indefinite for the use of the term "modified." Claim 17 has been amended to more distinctly claim the Applicant's invention, rendering this rejection moot.

#### Rejections Under 35 U.S.C. §102(e)

Claims 1, 2, 4, 6, 8, 9, 13, and 15 are rejected as allegedly being anticipated by Noonberg et al. (U.S. Patent 5,624,803). Claims 2 and 4 have been canceled. This rejection is traversed as applied to claims 1, 6, 8, 9, and 13. Applicant respectfully traverses this rejection.

Noonberg et al. describes methods and constructs for delivering antisense, triplex, and/or ribozyme oligonucleotides intracellularly using U6-type RNA pol III based constructs which are termed "oligonucleotide generators." The constructs of Noonberg et al. includes a pol III promoter, a stabilizing region on the 5' end which can be a hairpin, an antisense sequence, and a termination sequence, where the components can be transcribed by RNA polymerase III to

produce a transcript (see column 8, paragraph 1). A second similar hairpin may be, but is not necessarily at the end of the termination sequence.

The subject invention consists of a pol II based system, not a pol III based system, as disclosed by Noonberg et al. A single prior art reference properly anticipates an invention under 35 U.S.C. §102 only if every element of the claimed invention is identically shown in that reference (*In re Bond*, 910 F.d. 831, 15 USPQ2d 1566 (Fed. Cir. 1990). Since Noonberg et al. Does not teach each and every element of the claimed invention, it cannot anticipate the claimed invention. Accordingly, Applicant respectfully requests withdrawal of the rejection.

#### Rejections Under 35 U.S.C. §103

Claims 3 and 5 stand rejected under 35 U.S.C. §103 over Noonberg et al. (U.S. Patent 5,624,803). Applicants respectfully traverse the rejection.

Claim 3 limits the stem loop structures described in claim 1 to the U1 snRNA stem loops. Claim 5 limits the pol II promoter described in claim 1 to the U1 snRNA promoter.

As described above, Noonberg et al. disclose methods and constructs for delivering antisense, triplex, and or/ribozyme oligonucleotides intracellularly using U6-type RNA pol III based constructs. The subject invention consists of a pol II based system, not a pol III based system, as disclosed by Noonberg et al. Noonberg et al. teach away from the pol II based system; they describe a pol II based system as transcribing at a low rate, generating varying transcript lengths, and producing transcripts with long polyadenylated tails (seen as undesirable), and not being correctly localized in the cell (see the paragraph that bridges the end of column 17 to the beginning of column 18).

The discussion quoted in the Office Action from column 42 of the Noonberg patent, is directed toward monitoring genes for signs of transcriptional dysfunction, not toward the use of the U1 genes in the constructs of Noonberg et al. In this particular example, U1 is monitored in order to document if the U6 based construct specifically inhibits the target. Noonberg et al is describes the selection of U1 because it is nuclear, abundant, constitutively expressed, and stable. Applicant submits that other genes, such as  $\beta$ -actin, could just have easily been used. In the Example, U1 levels are not affected following transfection with the chimeric U6 gene, as expected.

Noonberg et al. also describe many differences between U1 and U6 (e.g., pol II vs. pol III transcription, the presence of a TATA box) in column 42. Although as Noonberg states, U1 is related to U6 as it can form a spliceosome, there are a great many differences between U1 and U6 Examples of these differences include: (1) U1 is more abundant than U6 (2) U1 utilizes RNA pol II, while U6 utilizes RNA pol III, (3) U1 has a 5' and a 3' stem loop structure, U6 has a single stem loop structure, (4) U1 has a trimethylguanosine cap, U6 has a  $\gamma$ -monomethylguanosine cap, (5) U1 is a member of the Sm class (a classification based on recognition by anti-Sm antibodies, all small RNPs known to participate in synthesis, processing, and export to cytoplasm of mature RNAs react with anti-Sm antibodies) while U6 is not, (6) U1 functions independently, U6 requires the presence of U4, (7) U1 is dispersed in the nucleoplasm, while U6 is concentrated in speckles and coiled bodies (Baserga and Steitz, 1993, "The Diverse World of Small Ribonucleoproteins," In: *The RNA World*, Cold Spring Harbor Laboratory Press, New York, pp. 359-381).

Consequently, Applicants submit that the use of U6 does not render the use of U1 obvious, and respectfully request withdrawal of the rejection.

Claims 1-6, 8-10, 12, 13, and 15 stand rejected over Michienzi et al., who describe a U1 snRNA vector for specifically targeting a ribozyme to the nucleus. Applicant respectfully traverses this rejection.

In the constructs of Michienzi et al. the catalytic core of the ribozyme's hammerhead motif is substituted into the stem-loop III of the U1 snRNA. This location is selected for very specific reasons delineated on page 7220, column 2, first paragraph. Applicants submit that the construct is shown in Figure 1, bottom left, on page 7221. The Office Action states that the construct is depicted in Figure 1, bottom right. Applicants submit that the bottom right portion of the Figure illustrates the pairing of Rev pre-mRNA with U1-Rz-5'. In the bottom right portion, mismatches are shown to occur within a single 5' stem loop structure of U1, and the cleavage sites for the ribozyme within the single, 5' stem loop are indicated (see the Figure legend).

All of the constructs taught by Michienzi et al. have substitutions in the 5' stem loop; no construct has unmodified stem loop structures, which are an essential component of the Applicant's invention. Applicant submits that a construct containing a unmodified, naturally occurring 5' U snRNA stem loop structure, and unmodified, naturally occurring 3' U snRNA stem loop structure, with an antisense sequence located between the two stem loops, would not be obvious from the work of Michienzi et al.

Furthermore, an antisense effect is not desired in the constructs of Michienzi et al.; specific constructs are designed to control for any antisense effect (p. 7220, column 2, paragraph 3). Therefore, Applicants submit that the substitution of the antisense construct for the ribozyme contained in the constructs of Michienzi et al. would not be obvious. Applicants respectfully request that the rejection be withdrawn.

Claims 1, 4, 6, 8, 9, 12, 13, and 15 are rejected over Taira et al. (U.S. Patent 5,500,357). Applicant respectfully traverses this rejection.

Taira et al. describe a recombinant DNA in which a DNA encoding a trans-acting ribozymes of interest is ligated to DNAs encoding another cis-acting ribozymes which serve to cleave the 5' and 3' ends of the trans-acting ribozyme of interest. The system is constructed by connecting the units in tandem. No stem loop structures are contained in the constructs of Taira et al. Applicants would like to point out that the structures shown in Figures 3 and 7b are ribozyme structures, not stem loop structures as suggested in the Office Action. Applicants submit that the tandem ribozyme constructs disclosed by Tara et al. do not render the use of 5' and 3' unmodified, naturally occurring U snRNP stem loops obvious for targeting antisense, and request withdrawal of the rejection.

Claims 1-6, 8-11, 13, and 15 stand rejected over De Young et al., who disclose the targeting of hammerhead ribozymes to atrial natriuretic factor mRNA using the construct pUHHU<sub>117</sub>, which contains the U1 promoter and the U1 3' end, which contains the terminator and the 3' stem loop structure (see page 12129, column 1, figure 3, and page 12136, column 1, last paragraph). Applicant respectfully traverses this rejection. The constructs of De Young et al. do not contain a 5' stem loop structure, nor do they suggest the positioning of an antisense sequence between two U snRNP stem loop structure.

Moreover, De Young et al. specifically state that "...in vitro transcription of the RNA polymerase II-activated U1 promoter is problematic..." (page 12129, column 2, paragraph 2), and they thus used a PCR based approach to produce chimeric DNA which encoded the T7 promoter, the U1 initiation sequence, and the ribozyme and U1 termination sequences. De Young et al. specifically teach away from the use of a pol II promoter, which is an inherent part

of the subject invention. The Applicant submits, therefore, that the work of De Young et al. does not render the subject invention obvious, and respectfully request withdrawal of the rejection.

In view of the comments above and the amendments to the claims, Applicants respectfully request the rejection be withdrawn.

Conclusion

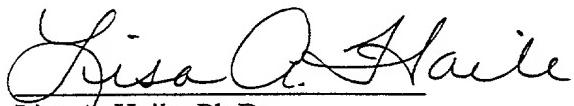
In summary, for the reasons set forth herein, Applicant submits that all of the pending claims are now in condition for allowance, which action is requested.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (619) 678-5070.

Please charge any additional fees, or make any credits, to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 8/18/97

  
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7388G PAL1/39100

PATENT  
ATTORNEY DOCKET NO. 07265/09802

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Harry C. Dietz                          Art Unit: 1804  
Serial No.: 08/742,943                              Examiner: D. Clark  
Filed : 10/31/96  
Title : DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS AND  
          METHODS OF USE

Assistant Commissioner for Patents  
Washington, DC 20231

**AMENDMENT**

In response to the Final Office Action dated November 17, 1997, in connection with the above-identified application, please consider the following amendments and remarks:

Date of Deposit January 20, 1998

I hereby certify under 37 CFR 1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

*Lucille M. Begalla*

Lucille M. Begalla

**In the Claims:**

1. (Amended) A nucleic acid construct for suppressing expression of a target gene, comprising:  
an antisense nucleic acid sequence directed to a target gene of interest;  
an unmodified, naturally occurring 5' U snRNA stem loop structure 5' of said antisense nucleic acid sequence [stem loop structure];  
a pol II promoter region 5' of said antisense [region] nucleic acid sequence; and  
3' of said antisense region [an unmodified,] a naturally occurring 3' U snRNA stem loop structure,  
wherein said expression of said target gene is suppressed by at least 75% of the normal level of expression .
3. (Reiterated) The nucleic acid construct of claim 1, wherein the U snRNA is U1.
5. (Reiterated) The nucleic acid construct of claim 1, wherein the promoter is a U1 snRNA promoter.
6. (Reiterated) The nucleic acid construct of claim 1, wherein the promoter is a constitutive promoter.
7. (Reiterated) The nucleic acid construct of claim 1, wherein the promoter is an inducible promoter.
8. (Amended) The nucleic acid construct of claim 1, further comprising a ribozyme nucleic acid which specifically cleaves mRNA transcribed from said target gene .

9. (Reiterated) The nucleic acid construct of claim 8, wherein the ribozyme nucleic acid is located between the 5' and 3' loop structures.
10. (Reiterated) The nucleic acid construct of claim 8, wherein the ribozyme nucleic acid is a hammerhead-type ribozyme.
11. (Reiterated) The nucleic acid construct of claim 8, wherein a consensus sequence for ribozyme cleavage in a target nucleic acid is 5'-GUC-3' or 5'-GUA-3'.
12. (Amended) The nucleic acid construct of claim 1, wherein the antisense nucleic acid is targeted to a region of a gene [is] selected from the group consisting of rent-1, HPV E6, HIV, hyaluronic acid synthase, and fibrillin.
13. (Amended) A method for suppression of gene expression comprising administering to a cell [a suppressive-effective amount of] the nucleic acid construct of claim 1.
15. (Reiterated) The method of claim 13, wherein the administering is *in vitro*.
17. (Reiterated) The method of claim 13, further comprising:  
administering a second nucleic acid encoding a wild-type polypeptide corresponding to the gene product of the gene being suppressed, wherein the second nucleic acid is resistant to ribozyme cleavage and/or antisense inhibition.
20. (Reiterated) The nucleic acid construct of claim 1, further comprising a 5' trimethylguanosine cap.

#### REMARKS

Claims 1, 3, 5-13, 17 and 20 are pending. Claims 1, 8, 12, and 13 are amended. Support for the amending language of claim 8 can be found in the specification on page 10, lines 3-10.

## Summary of the Invention

The subject invention relates to a novel nucleic acid construct for delivery of antisense sequences for suppressing target gene expression. The construct includes a pol II promoter region, a 5' and a 3' unmodified U snRNA stem loop structure, and an antisense region located between the 5' and the 3' stem loop structures. Optionally, a ribozyme and/or a 5' trimethylguanosine cap are included in the construct.

## Rejection Under 35 U.S.C. §112, first paragraph

Claims 8-11 stand rejected under 35 U.S.C. §112, first paragraph as the breadth of the claims allegedly renders them non-enabled. The Office action states that amendment to point out the relationship of the ribozyme to the gene of interest will provide enablement for the claims. Claim 8 has now been amended to recite that the ribozyme specifically cleaves mRNA transcribed from said target gene. Claims 9-11 depend from claim 8. Applicants submit that the amendment to claim 8 renders the rejection moot.

## Rejections Under 35 U.S.C. §112, second paragraph

The Office action states that the previously made rejection to the terms “unmodified” and “suppressive-effective” is withdrawn, but recommends removal of the term. In order to more clearly define the invention, and to advance prosecution of the application, the term “unmodified” has been removed from claim 1 and the term “suppressive-effective” is removed from claim 13, as recommended.

Claim 1 is rejected for the recitation of “of said antisense nucleic acid stem loop structure” in lines 4-5 of the claim. Claim 1 has been amended, rendering the rejection moot.

Claim 12 is rejected for the recitation of “wherein the antisense nucleic acid is targeted to a region of a gene is selected from...”. Claim 12 has been amended to delete the second recitation of the word “is,” as recommended. Applicants submit that this amendment renders the rejection moot.

Claims 3, 5-13, 15, 17, and 20 are rejected as being dependent of rejected claims 1 and 12. Applicants submit that the amendments to claim 1 and 12 render this rejection moot.

## CONCLUSION

In summary, for the reasons set forth herein, Applicant submits that all of the pending claims are now in condition for allowance, which action is requested.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (619) 678-5070.

Please charge any additional fees, or make any credits, to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 1/20/98



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**DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS  
AND METHODS OF USE**

5

**Field of the Invention**

This invention relates generally to the field of delivery vehicles for nucleic acid molecules and specifically to a novel construct for delivery of antisense targeting sequences to a cell.

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**Background of the Invention**

The use of antisense oligonucleotides offers advantages over other therapeutic regimes due to their potential for target specificity. For example, conventional chemotherapy for neoplastic and virus-related diseases has the disadvantage of systemic toxicity. The therapeutic index for chemotherapeutic agents is relatively narrow, since such agents are unable to distinguish between normal and diseased cells. Antisense oligonucleotides have the potential to be many orders of magnitude more specific than traditional drugs due to their greater number of interactions with a particular target site. In theory, an oligonucleotide of more than 15-17 nucleotides in length could have the base pairing specificity to interact with only one target gene within the entire human genome. Thus, antisense oligonucleotides have the potential specificity that could serve as a powerful tool for the study of specific gene function and as therapeutic agents for disease-causing genes.

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In contrast to drugs, antisense molecules are relatively simple to design. The interaction between an antisense oligonucleotide and a target mRNA is governed primarily by the sequence of the target. Oligonucleotides targeting the start codon and extending upstream or downstream have been shown to be effective. Similarly, oligonucleotides that are complementary to the splice sites have proved effective.

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Antisense technologies for the targeted inhibition of gene expression could provide an effective strategy for the management of inherited disorders with dominant-negative or gain-of-function pathogenetic mechanisms, for the suppression of oncogenes, or for the control of a variety of infectious agents. Pathologic disorders that are currently targeted by antisense therapeutics include viral infections, inflammatory disorders, cardiovascular disease, cancers, genetic disorders and autoimmune diseases. Synthetic oligodeoxynucleotides (ODNs), especially phosphorothiates and methylphosphonates, offer the advantage of enhanced stability in biological fluids and an effectively limitless supply.

Antisense oligonucleotides are also useful for the production of transgenic animals having alterations at the germline level, such as knockout mutations, which can be used for the study of new genes or the study of the function of a known gene. Further, antisense technology combined with gene therapy is useful for example, for suppression of expression of a mutant gene product. Such gene therapy would be most advantageous in combination with a replacement regimen utilizing the "normal" gene to provide a "normal" gene product.

Unfortunately, the effective use of antisense oligonucleotides has been limited due to several problems. Disadvantages include the transient nature of ODNs, and their toxicity and propensity for producing non-sequence specified biological effects. Other disadvantages include low expression or limited stability of complementary RNAs which result in their nonspecific targeting or low efficiency of target inhibition. Antisense oligonucleotides are often poorly taken up by cells and therefore may never reach their target site. Often, antisense oligonucleotides do not reach the nucleus of a cell once administered, the site of their RNA and DNA targets. In certain applications the antisense molecules are microinjected directly into the cells. This technique works well in the laboratory, however, it cannot be applied to patients. Many of the studies with antisense show that gene expression is suppressed by 80-90% of the normal level,

however, such reduction is not typically sufficient to reduce the biological effect, *e.g.*, 10-20% expression is sufficient to maintain the biological function sought to suppress.

There is a need to develop a delivery system for antisense molecules that gives the antisense enhanced stability, for example by being resistant to nuclease activity or by being enriched in the nucleus, while still allowing specificity of the antisense for its target RNA or DNA. Such a system would provide effective targeting of the message with the end result being significant inhibition of expression of a particular gene.

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### Summary of the Invention

The present invention provides a novel nucleic acid construct for delivery of antisense targeting sequences for inhibition of selected genes in a cell. The construct includes intact stem loop structures and an antisense nucleic acid. This allele-specific targeting is also useful in combination with replacement gene therapy.

In a first embodiment, the invention provides a nucleic acid construct for suppressing gene expression. The construct includes a 5' stem loop structure, an antisense nucleic acid, and a 3' stem loop structure. Such "unmodified" or "intact" stem loop structures flank the antisense nucleic acid so that the antisense oligonucleotides can readily interact with any target sequence. In a preferred embodiment, the stem loop structures are U snRNA stem loops, and most preferably, U1 snRNA stem loops. The construct provides a cloning site outside of the stem loop structures, thus rendering the stem loop structures "unmodified," into which virtually any antisense oligonucleotide could be inserted. In contrast to previous antisense delivery vehicles which have been designed to interfere with mRNA splicing, the construct of the present invention does not necessarily affect the splicing machinery so as not to disrupt normal cellular mRNA processing. In other words, while previous constructs retain elements that interact with spliceosome proteins, the construct of the invention eliminates such elements. Preferably, a U1 snRNA promoter is included in the construct. Optionally, a ribozyme nucleic acid is included in the construct.

In another embodiment, the invention provides a method for suppression of gene expression whereby a suppressive-effective amount of the nucleic acid construct of the invention is administered to a cell thereby suppressing expression of the gene. The method is particularly useful when used in combination with replacement gene therapy which provides a gene which encodes and can reconstitute the wild-type protein and which is resistant to targeting by the antisense and/or ribozyme.

The seminal discovery of the delivery vehicle of the invention now allows transgenic knockout animals to be created for the study of known or as of yet unknown

genes. For example, phenotypic consequences of loss of function due to targeted disruption of a gene(s) can be determined.

Brief Description of the Drawings

Figure 1 shows an illustrative expression construct of the invention.

Figure 2 shows the sequence and predicted structure of chimeric transcripts derived from pU1/FIB.

5       Figure 3 shows an immunohistochemical analysis of MG63 cells that were stably transfected with an expression construct (pZeoSVLacZ, Invitrogen) lacking targeting sequence (panels A and C) or pU1/FIB (panels B and D).

Figure 4 shows a Northern blot analysis of 3.5 µg of poly(a) RNA extracted from confluent MG63 cells that were untransfected (lane 1), or stably transfected with either pZeoSVLacZ (Lane 2) or pU1/FIB (lane 3-5).

Description of the Preferred Embodiments

The present invention provides a novel nucleic acid construct which provides a general means of delivery for antisense targeting sequences. The construct includes stabilizing structural elements, stem loop structures, which increase the effectiveness of the targeting construct. Also included is a method of suppression of gene expression utilizing the novel construct of the invention.

The nucleic acid construct of the invention which is useful for suppressing gene expression includes 5' and 3' stem loop structures and an antisense nucleic acid. While not wanting to be bound by a particular theory, it is believed that the stem loop structures provide enhanced stability of the targeting molecules by conferring resistance to exonucleases and also promote duplex formation and stability.

The "stem loop" structures refer to nucleic acid structures that have folding patterns which form hairpins and flank the antisense targeting sequence. The stem loop structures are preferably unmodified, naturally occurring structures. Alternatively, one of skill in the art would be able to synthesize such structures to "mimic" the naturally occurring structures. The preferred stem loop structures in the construct of the invention are unmodified U snRNA structures. The term "unmodified" means that the folding pattern of the stem loop structure is not compromised by alterations in the nucleic acid sequence of the naturally occurring molecule. For example, it is understood that alterations which include, but are not limited to, mutations, insertions, deletions and substitutions of one or more nucleotides can be made within the sequence of the stem loop, as long as the stabilization function and hairpin formation of the stem loop is maintained.

Preferably, the stem loop structures are derived from or synthesized according to the nucleotide sequence of the U snRNA stem loop structures. An exemplary construct utilizing U1 snRNA stem loops is shown in FIGURE 1 herein. One can produce a chimeric construct having a 5' stem loop structure from one species and a 3' structure

from a second species. For example, a 5' stem loop is derived from human U snRNA and the 3' stem loop derived from a yeast U snRNA. Other snRNA stem loop structures can be derived from other mammals (e.g., porcine, bovine), or yeast for example. Other chimeric constructs can be prepared using a 5' stem loop structure from one snRNA (e.g., 5 U1) and a 3' stem loop structure from another snRNA (e.g., U3). Virtually any combination of stem loop structures is envisioned in the construct of the invention.

As used herein, the term "nucleic acid" or nucleic acid sequence" refers to a polymer of deoxyribonucleotides or ribonucleotides, in the form of a separate fragment or as a component of a larger construct. For example, nucleic acids can be assembled from cDNA fragments or from oligonucleotides to generate a synthetic gene which is capable of being expressed in a recombinant transcriptional unit. Polynucleotide or nucleic acid sequences of the invention include DNA, RNA and cDNA sequences.

Nucleic acid sequences utilized in the invention can be obtained by several methods. Sequences for specific genes or stem loop structures can determined from published sequences and can also be found in GenBank, National Institutes of Health computer database. Nucleic acids can then be chemically synthesized by standard methods for example.

The construct of the invention includes an antisense nucleic acid flanked by the stem loop structures. The antisense nucleic acid can be directed toward any target nucleic acid, and preferably to a target message (mRNA). Any antisense which includes sequences capable of hybridizing with its complementary target can be used in the construct of the invention. For example, antisense sequences can be directed to the 5' or 20 3' termini of the target message, to splice junctions, or to internal sequences. One of skill in the art will readily be able to determine which sequences to use as the appropriate antisense nucleic acid construct. "Antisense nucleic acids" are DNA or RNA molecules 25 that are complementary to at least a portion of a specific mRNA molecule (Weintraub, Scientific American, 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic

acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to be toxic than larger molecules when introduced into the target cell. The use of antisense methods to inhibit the *in vitro* 5 translation of genes is well known in the art (Marcus-Sakura, *Anal. Biochem.*, 172:289, 1988). An antisense core nucleic acid may contain about 10 nucleotides complementary to the target message. Examples of target messages include transcription regulatory factors (e.g., rent-1), viral encoded proteins (e.g., human papilloma virus E6, human immunodeficiency virus tat), (e.g., haluronic acid synthase), structural proteins (e.g., fibrillin), cytokines, oncogenes and growth factors (e.g., interleukins), etc. It should be noted that the antisense nucleic acid can be useful for reducing the expression of either 10 normal or aberrant genes.

The antisense nucleic acid can be used to block expression of a mutant protein or a dominantly active gene product, such as amyloid precursor protein that accumulates 15 in Alzheimer's disease. Such methods are also useful for the treatment of Huntington's disease, hereditary Parkinsonism, and other diseases. Antisense nucleic acids are also useful for the inhibition of expression of proteins associated with toxicity or gene products introduced into the cell, such as those introduced by an infectious agent (e.g., a virus).

It may be desirable to transfer an antisense nucleic acid encoding a biological response modifier in order to reduce the expression of such biological response modifier. Included in this category are nucleic acids encoding immunopotentiating agents including 20 a number of the cytokines classified as "interleukins". These include, for example, interleukins 1 through 12. Also included in this category, although not necessarily working according to the same mechanisms, are interferons, and in particular gamma interferon ( $\gamma$ -IFN), tumor necrosis factor (TNF) and granulocyte-macrophage-colony stimulating factor (GM-CSF). It may be desirable to deliver such antisense nucleic acids 25 to cells of the immune system to treat enzymatic related disorders or immune defects.

Antisense nucleic acids can be used to reduce expression of growth factors, toxic peptides, ligands, receptors, or other physiologically important proteins.

The construct of the invention may further include a ribozyme nucleic acid.

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, *J.Amer.Med. Assn.*, 260:3030, 1988). A major advantage of this approach is that, because the ribozymes are engineered to be sequence-specific, only mRNAs with sequences complementary to the construct containing the ribozyme are inactivated.

There are two basic types of ribozymes namely, tetrahymena-type (Hasselhoff, et al., *Nature*, 334:585, 1988) and "hammerhead"-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences from about 3 to 18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating a specific mRNA species. The preferred ribozyme of the present invention is a hammerhead type ribozyme. The target message is required to contain a ribozyme cleavage site sequence such as 5'-GUC-3' or 5'-GUA-3' when a ribozyme is included in the construct.

The construct may also optionally include a triplex oligomer. Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, et al., *Antisense Res. and Dev.*, 1(3):227, 1991; Helene, C., *Anticancer Drug Design*, 6(6):569, 1991).

The phrase "nucleic acid sequence expressing a product of interest" refers to a nucleic acid sequence which when expressed results in a product selected from a protein

or antisense RNA, for example. The term "structural gene" excludes the non-coding regulatory sequence which drives transcription. The structural gene may be derived in whole or in part from any source known to the art, including a plant, a fungus, an animal, a bacterial genome or episome, eukaryotic, nuclear or plasmid DNA, cDNA, viral DNA or chemically synthesized DNA. A structural gene may contain one or more modifications in either the coding or the untranslated regions which could affect the biological activity or the chemical structure of the expression product, the rate of expression or the manner of expression control. Such modifications include, but are not limited to, mutations, insertions, deletions and substitutions of one or more nucleotides.

The structural gene may constitute an uninterrupted coding sequence or it may include one or more introns, bound by the appropriate splice junctions.

The term "operably associated" refers to functional linkage between the regulatory (e.g., promoter) sequence and the nucleic acid regulated by the regulatory sequence. The operably linked regulatory sequence controls the expression of the product expressed by the structural gene or antisense. The regulatory sequence may be homologous or heterologous to the desired gene sequence. A wide range of promoters may be utilized, including viral or mammalian promoters. Cell- or tissue-specific promoters can be utilized to target expression of gene sequences in specific cell populations. Suitable mammalian and viral promoters for the present invention are available in the art. A preferred promoter in the construct of the invention is a U snRNA promoter, and most preferably, a U1 snRNA promoter.

The choice of a particular heterologous promoter region as a "regulatory nucleotide sequence" of the invention, is dictated by the spatial and temporal pattern of expression that is desired for the transactivator gene and ultimately for the target transgene. Promoter regions of the invention include eukaryotically derived promoters which predominantly direct expression in, for example, the reproductive system (e.g., breast, ovary, testes); the musculoskeletal system (e.g., muscle or joint tissue); the cardiovascular system (e.g., capillaries or heart); the respiratory system (e.g., lung or

nasal passages); the urological system (*e.g.*, kidney or bladder); the gastrointestinal system (*e.g.*, pancreas, liver, or intestines); the immune system (*e.g.*, thymus, spleen, or circulating immunological cells); the endocrine system (*e.g.*, pituitary, gonads, and thyroid); the nervous system (*e.g.*, neurons); and the hematopoietic system (*e.g.*, bone marrow and peripheral blood). Further, promoters of the invention include, but are not limited to the elastase promoter (including its enhancer; expression in pancreatic acinar cells); the alpha-A-crystallin promoter (expression in the eye lens tissue); the insulin promoter (including enhancer; expression in the pancreatic beta cells) and the albumin promoter region, including its enhancer.

Alternatively, non-eukaryotically-derived promoters such as virally-derived and prokaryotically-derived promoters are also included in the present invention. Such virally-derived promoters include, but are not limited to MMTV and MoSV LTR, SV40 early region, RSV or CMV LTR, which direct expression of viral or host genes in specific tissues and in many cell types.

Promoters useful in the invention include both constitutive and inducible natural promoters as well as engineered promoters. To be most useful, an inducible promoter should 1) provide low expression in the absence of the inducer; 2) provide high expression in the presence of the inducer; 3) use an induction scheme that does not interfere with the normal physiology of the cell; and 4) have no effect on the expression of other genes. Both constitutive and inducible promoters and enhancers will be known to those of skill in the art. The promoters used in the construct of the present invention may be modified, if desired, to affect their control characteristics.

The regulatory nucleotide sequence of the invention may also include “enhancer” regions. Enhancers, as used herein, refer to DNA sequences which affect transcription of a gene by RNA polymerase II, without regard to position or orientation. An enhancer region can be thousands of base pairs from a transcription unit and still affect its transcription. Examples of enhancers known in the art include the

immunoglobulin heavy (mu) chain or light (kappa) chain enhancers and the SV40 72-base pair repeats.

It may be desirable to deliver the construct of the invention directly to the cell without the requirement for expression, therefore, a promoter or other regulatory nucleic acid is optional. One example of a targeted delivery system for antisense polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes or liposome formulations. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. These formulations may have net cationic, anionic or neutral charge characteristics are useful characteristics with *in vitro*, *in vivo* and *ex vivo* delivery methods. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0  $\mu\text{m}$  can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al.*, *Trends Biochem. Sci.*, **6**:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.*, *Biotechniques*, **6**:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used.

The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

5           The construct of the invention can also be delivered as a naked “gene expression vector”. This means that the construct is not associated with a delivery vehicle (e.g., liposomes, colloidal particles and the like). One of the principal advantages touted for naked DNA vectors has been the lack of immune responses stimulated by the vector itself.

10           The backbone or framework of the construct preferably includes U1 snRNA nucleic acid sequences since U1 snRNA is enriched in the nucleus, is widely dispersed in the nucleoplasm, and is abundantly expressed. In the exemplary construct shown in FIGURE 1, an “antisense targeting core” was substituted for the Sm protein binding site between the two naturally occurring U1 snRNA hairpins. This core contains a 30-35 basepair sequence that is directly complementary to the target message, interrupted in its center by an autocatalytic hammerhead ribozyme loop. Duplex formation aligns the ribozyme with the GUC or GUA consensus sites for ribozyme cleavage within the target message. An illustrative expression construct of the invention which targets RENT1 transcripts, which are involved in the NMRD pathway, described below, is shown in Figure 1. The term rent1 and RENT1 (regulator of nonsense transcripts) refers to the gene and protein, respectively, from either the murine or human species.

20           The construct described above is useful for suppressing gene expression. For example, it may be desirable to modulate the expression of a gene when it is over-expressed. Where a cell proliferative disorder is associated with the expression of a gene, nucleic acid sequences that interfere with the gene’s expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid, ribozymes, or triplex agents to block transcription or translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme, as described above.

The construct may also optionally include a 5' cap structure, such as a trimethylguanosine cap.

In another embodiment, the present invention also includes a method for suppression of gene expression which includes administering to a cell a suppressive-effective amount of the nucleic acid construct of the invention so that the expression of the gene is suppressed. The term "suppressive-effective" amount means that amount of the construct, and thus antisense, administered is sufficient to suppress the expression of the target, *e.g.*, inhibit translation of mRNA, by at least 75% of the normal expression, and preferably by at least 90%. The effectiveness of the construct can be determined phenotypically or by standard Northern blot analysis or immunohistochemically, for example. Other standard nucleic acid detection techniques or alternatively immunodiagnostic techniques will be known to those of skill in the art (*e.g.*, Western or Northwestern blot analysis).

The present invention also provides a method of gene therapy for the treatment of cell proliferative or immunologic disorders and diseases such as which are mediated by various proteins. The term "cell-proliferative disorder" denotes malignant as well as non-malignant cell populations which often appear to differ from the surrounding tissue both morphologically and genotypically. Such disorders may be associated, for example, with abnormal expression of a gene. "Abnormal expression" encompasses both increased or decreased levels of expression, as well as expression of a mutant form of a gene such that the normal function of the gene product is altered. Abnormal expression also includes inappropriate expression of during the cell cycle or in an incorrect cell type. The antisense polynucleotide is useful in treating malignancies of the various organ systems. Such therapy would achieve its therapeutic effect by introduction of the antisense construct into cells having the proliferative disorder. Delivery of antisense polynucleotide, can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system as described above.

The construct may also be useful in treating malignancies of the various organ systems, such as, for example, lung, breast, lymphoid, gastrointestinal, and genito-urinary tract as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer, leukemia, breast cancer, non-small cell carcinoma of the lung, cancer of the small intestine, and cancer of the esophagus.

The method is also useful in treating non-malignant or immunologically-related cell-proliferative diseases such as psoriasis, pemphigus vulgaris, Bechet's syndrome, acute respiratory distress syndrome (ARDS), ischemic heart disease, post-dialysis syndrome, rheumatoid arthritis, acquired immune deficiency syndrome, vasculitis, lipid histiocytosis, septic shock and inflammation in general.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, adeno-associated virus, herpes virus, vaccinia, or, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). Preferably, when the subject is a human, a vector such as the gibbon ape leukemia virus (GaLV) is utilized. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector containing the antisense polynucleotide.

Administration of the construct of the invention can be *in vivo*, *in vitro* or *ex vivo*.

The method of the invention also envisions gene replacement therapy to reconstitute expression of a wild-type protein in cells expressing a defective protein or, in the case of a heterozygote, a defective and a "normal" protein. Therefore, in another embodiment the invention provides a method of suppressing gene expression as above and further comprises administering a modified nucleic acid encoding a wild-type polypeptide corresponding to the gene product of the gene being suppressed, wherein the modified nucleic acid is resistant to ribozyme cleavage and/or antisense inhibition. Due to degeneracy in the genetic code, multiple nucleotide substitutions can be made across the antisense oligonucleotide target region of the coding sequence within an exogenously supplied expression construct encoding the wild-type gene of interest. Such substitutions would inhibit duplex formation between the gene suppressed and the antisense nucleic acid and transcripts derived from the replacement "normal" gene, while maintaining the fidelity of the amino acid sequence. In other words, the replacement gene would provide a protein with the correct amino acid sequence utilizing codons with nucleotides that are not recognized by the antisense construct and/or ribozyme. In this method, it is therefore possible to "knockout" a defective copy of a gene/mRNA and introduce a "normal" copy resistant to the "knockout" construct. Thus, both normal and aberrant expression of a gene can be blocked by introduction of the antisense construct of the invention; to restore normal function of the gene, a copy of the wild-type gene is introduced, that, while coding for a normal protein, is resistant to duplex formation with the antisense construct and/or ribozyme cleavage.

There are a number of inherited diseases in which defective genes may be replaced including: lysosomal storage diseases such as those involving  $\beta$ -hexosaminidase or glucocerebrosidase; deficiencies in hypoxanthine phosphoribosyl transferase activity (the "Lesch-Nyhan" syndrome); amyloid polyneuropathies (prealbumin); Duchenne's muscular dystrophy, and retinoblastoma, for example.

Pathologic disorders that can be targeted using the method of the invention include but are not limited to viral infections, inflammatory disorders, cardiovascular disease, cancers, genetic disorders and autoimmune diseases.

The method of the present invention is also useful for suppression of a dominant negative mutation. The term "dominant negative" is a term of art and refers to expression of a gene resulting in a gene product that actively interferes with the function of a "normal", endogenous protein. Thus, a mutant protein or dominantly active gene product, such as amyloid precursor protein that accumulates in Alzheimer's disease can be blocked. The dominant negative phenotype is conveyed by the expression of the mutant protein that interferes with the function of the normal protein. Such an effect is similar to the effect of dominance of one allele of a pair of alleles encoding homologous genes on a pair of homologous chromosomes such that the phenotypic effect of the one allele exerts a deleterious controlling influence over the other allele.

The nucleic acid construct can be utilized in the discovery of unknown human genes, for example, with *a priori* knowledge of the gene in question. In one embodiment of this invention, cells are isolated from an individual displaying a particular phenotype which is suspected of being caused by a gene or genes containing nonsense mutations. A subset of these cells is contacted (e.g., by transfecting the cells), with the construct that contains at least an antisense nucleic acid for suppression or inhibition of the expression or function of a gene (e.g., Rent-1 gene or other genes involved in NMRD) or its gene product in the cells. Levels of mRNA containing nonsense mutations in these cells are elevated to higher levels. Using standard techniques of differential display of mRNA, or genetic subtraction techniques commonly applied to cDNAs, the mRNA containing nonsense mutations can be enriched and then isolated by comparing the mRNA populations of the original cells with the mRNA populations in cells containing the antisense.

In a further embodiment, a transgenic animal can be developed using the novel construct and method of the invention in order to identify the impact of increased or

decreased gene expression on a particular pathway or phenotype. Protocols useful in producing such transgenic animals are described below. The protocol generally follows conventional techniques for introduction of expressible transgenes into mammals. Those of ordinary skill in the art will be familiar with these applications and will be able to apply the techniques in the context of the present invention without undue experimentation.

For example, embryonic target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonic target cell. The zygote is the best target for microinjection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2pl of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster, *et al.*, *Proc. Natl. Acad. Sci. USA* 82:4438-4442, 1985). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. In general, this will also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. Microinjection of zygotes is a preferred method for incorporating transgenes in practicing the invention.

Retroviral infection can also be used to introduce transgene into a non-human animal. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenisch, *Proc. Natl. Acad. Sci USA* 73:1260-1264, 1976). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan, *et al.*, *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner, *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:6927-6931, 1985; Van der Putten, *et al.*, *Proc. Natl. Acad. Sci USA*

82:6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Steward, *et al.*, *EMBO J.*, 6:383-388, 1987).

5 Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner, *et al.*, *Nature*, 298:623-628, 1982). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (Jahner, *et al.*, *supra*, 1982).

10 A third type of target cell for introduction of heterologous nucleic acid sequences is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans, *et al.*, *Nature*, 292:154-156, 1981; Bradley, *et al.*, *Nature*, 309:255-258, 1984; Gossler, *et al.*, *Proc. Natl. Acad. Sci USA*, 83:9065-9069, 1986; and Robertson, *et al.*, *Nature*, 322:445-448, 1986).

15 Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. These transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells will thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal (see for review, Jaenisch, *Science*, 240:1468-1474, 1988). Any ES cell may be used in accordance with the present invention. It is, however, preferred to use primary isolates of ES cells. Such isolates may be obtained directly from embryos such as with the CCE cell line disclosed by Robertson, E.J., in Current Communications in Molecular biology, Capecchi,M.R. (Ed.) Cold Springs Harbor Press, Cold Springs Harbor, NY (1989), pp.39-44), or from the clonal isolation of ES cells from the CCE cell line (Schwartzberg, P.A. et al., *Science* 246:799 (1989). ES cells may be derived or isolated from any species, although cells

derived or isolated from mammals such as rodents, rabbits, and non-human primates are preferred.

Construction of transgenes can be performed by those of ordinary skill in the art using the teachings herein. One of ordinary skill in the art can "knock out" a gene in mice by targeted disruption of the gene. This can be accomplished by homologous recombination in murine embryonic stem (ES) cells using standard techniques. The clinical and cellular consequences of targeted disruption can be investigated in multiple genetic backgrounds including inbred strains, strains with many undefined nonsense alleles, and strains of known mutant genotype to determine if: (a) targeted disruption of the gene can effect a loss of function; (b) loss of function can have an phenotype consequences (i.e., the creation of knockout phenotypes), (c) loss of the gene product unmasks the effects of protein that can be expressed from "physiologic" somatically acquired or inherited nonsense alleles upon transcript stabilization.

The cre/lox system as described in US Patent Serial No. 4,959,317, incorporated herein by reference, can be utilized in the production of transgenic animals. A first and second loxP DNA sequence is introduced into cells connected by a pre-selected antisense or replacement gene, herein referred to as a "target transgene". The "target transgene" of interest can be a complete gene or any other sequence of nucleotides including those of homologous, heterologous, or synthetic origin. The target transgene sequence can be for example, an antisense or replacement gene for a structural protein, an enzyme, or a regulatory molecule. The target transgene may also be a gene of undetermined function. Using tissue-specific or developmentally-specific regulatory sequences (as described above) to direct expression of the target transgene, a function could be identified. If the first and second lox sites have the same orientation (direct repeats), activation of the regulatory nucleotide sequence of the transactivator transgene results in a deletion of the target transgene DNA, such that ablation or modification of activity results. If the first and second lox sites have opposite orientation (inverted

repeats), activation of the regulatory nucleotide produces an inversion of the nucleotides sequence of the target transgene.

The construct of the invention may be used to introduce DNA sequences into the germ line cells of "non-humans" to create transgenic animals. The preferred animal of the invention is a mouse. However, other non-humans of the invention include but are not limited to other rodents (e.g. rat, hamster), rabbits, chickens, sheep, goats, fish, pigs, cattle, and non-human primates.

In yet another embodiment, an antisense library can be constructed using the nucleic acid construct of the invention in order to identify novel genes. For example, a U1 construct library containing antisense core sequences (e.g., random sequences or sequences derived from a cDNA library) is transfected into host cells and clones exhibiting a desired phenotype are identified. Such clones are then examined to determine the nature of the regulatory sequence in the construct. Therefore, novel genes having specific functions can be identified. For example, cells that acquire a transformed phenotype may contain an antisense molecule for a novel tumor suppressor gene. A match between a given suppressive nucleic acid and a previously cloned gene can be made using standard techniques (e.g., BLAST search algorithm).

The following Examples are intended to illustrate, but not to limit the invention. While such Examples are typical of those that might be used, other procedures known to those skilled in the art may alternatively be utilized.

## EXAMPLES

The following examples provide a description of construction of an exemplary nucleic acid construct of the invention, utilizing 5' and 3' unmodified stem loop structures from U1 snRNA which flank an antisense molecule directed toward the fibrillin-1 gene (mRNA).

### EXAMPLE 1

An antisense expression construct was constructed that incorporates several potentially enhancing features. The pU1/FIB vector was constructed on the backbone of the pZeoSV (Invitrogen) prokaryotic/eukaryotic expression vector. The SV40 promoter, polyadenylation site and polylinker were excised from pZeoSV at the BamHI sites. A U1 snRNA expression cassette cloned into pUC13 was excised with BamHI digestion and ligated into the BamHI sites of the modified pZeoSV. Two rounds of site-directed mutagenesis (Deng, et al., *Anal. Biochem.*, 200, 81; 1992) were then performed to change four nucleotides flanking the Sm protein restriction sites (pZeoU1EcoSpe).

Complementary oligonucleotides that encode the antisense ‘core’ sequence, shown in Figure 1, including the 24 highly conserved nucleotides of hammerhead ribozymes (Cech, et al., *Ann. Rev. Biochem.*, 55:599, were synthesized and annealed at 40° C such that the remaining 5' and 3' overhangs were exactly complementary to the overhangs left by EcoRI and SpeI digestion. The sequences of the oligonucleotides were as follows:

5'-AATTGGCGATCTCCAGCACTGATGAGTCCGTGAGG  
ACGAAACGCCCTCGACGCAT-3' (SEQ ID NO:1),  
5'-CTAGATGCGTCGAGGGCGTTCGTCCTCACGGACTC  
ATCAGTGCTGGAGATGCC-3' (SEQ ID NO:2) (sense and antisense, respectively).

The resulting duplex was ligated into the EcoRI and SpeI sites of pZeoU1EcoSpe to

create pU1FIB. All ligation junctions were sequenced to verify the identity and orientation of the insert.

In that the structure described for naturally occurring antisense RNAs is highly similar to that for small nuclear RNAs (snRNAs), essential components of the spliceosome complex that are abundant and stable in the nucleus of mammalian cells, the U1 snRNA gene was selected as the framework for vector construction (Guthrie, et al., *Annu. Rev. Genet.*, 22; 387, 1988). Other attributes include the potent and constitutively active nature of the U1 snRNA promoter, the ability of the unusual trimethylguanosine 5' cap and Sm protein interactions to signal transport of U1 snRNA back into the nucleus (Hamm, et al., *Cell*, 62; 569, 1990; Fischer, et al., *Science* 249; 786, 1990; Fischer, et al., *J. Cell Biol.* 113 Mo; 705, 1991; Plessel, et al., *Mol. Cell. Biol.* 14, 4160, 1994), and the lack of polyadenylation of mature snRNAs, a factor which may favorably influence transcript trafficking and localization (Zhong, et al., *Proc. Natl. Acad. Sci. USA*, 91; 4258, 1994). Moreover, unlike other spliceosome components, U1 snRNA is widely dispersed in the nucleoplasm (Carmo-Fonseca, et al., *EMBO J.*, 10; 195, 1991; Matera, et al., *J. Cell. Biol.*, 121; 715, 1993).

As an illustrative example, the antisense targeting ‘core’ was designed to contain sequences exactly complementary to coding nucleotides 1-15 and 17-30 of fibrillin mRNA (Corson, et al., *Genomics*, 17; 476, 1993; Pereira, et al., *Hum. Mol. Genet.*, 2; 961, 1993) separated by the 22 bp hammerhead ribozyme loop. Dominant-negative forms of fibrillin-1 cause Marfan syndrome, an autosomal dominant systemic disorder of connective tissue (Dietz, et al., *Nature*, 352; 337, 1991). The regions of complementarity were predicted to align the autocatalytic structure with the consensus sequence for ribozyme cleavage (5'-GUC-3') within the target message (Cech, et al., *Annu. Rev. Biochem.*, 55; 599, 1986; T.R. Cech, *Science*, 236; 1532, 1987; T.R. Cech, *Ann. Rev. Biochem.*, 59, 543, 1990; Haseloff, et al., *Nature*, 334; 585, 1988). This antisense targeting sequence was substituted for the short Sm protein binding sequence

between the two hairpin loops of U1 snRNA (Guthrie, *Annu. Rev. Genet.*, 22; 387, 1988.) The sequence of the resulting chimeric RNA (Figure 1) was analyzed using a program that predicts RNA structure (Mike Zuker's RNA page: <http://ibc.wustl.edu/~zuker/rna/>). The boundaries of the targeting sequence utilized in the construct were selected to 5 maximize preservation of the U1 snRNA stem-loops, the ribozyme secondary structure and the accessibility of the sequence complementary to the target message. Expression of the chimeric RNA was under the transcriptional control of a region of U1 snRNA 5'- flanking sequence that has been shown to be potent and constitutively active (Zhuang, et al., *Cell*, 46; 827, 1986; Maugin, et al., *EMBO J.*, 5; 987, 1986); and Asselbeyers, et al., 10 *Mol. Biol. Rep.* 17; 101, 1993).

Figure 2 shows the sequence and predicted structure of chimeric transcripts derived from pU1/FIB. The Sm protein binding site of U1 snRNA (boxed) has been substituted by an antisense targeting core (flanked by dashed lines) that is complementary to the first 30 coding nucleotides of FBN1 and contains a hammerhead ribozyme sequence in its center. The two stem-loop structures of U1 snRNA are maintained. 15 Cleavage of the target message (unbolded characters) is predicted to occur immediately following the consensus sequence 5'-GUC-3' (arrow). The 5' cap structure of the chimeric targeting molecule may contain 1 or 3 methyl (m) groups. A sequence in the chimeric molecule (5'-AAUUGG-3', underlined) remains highly similar to the consensus 20 for Sm protein binding, PuA(U)nGPu (Pu = A or G).

A human osteosarcoma (MG63) cell line was stably transfected with either a reporter gene expression vector (pZeoSVLacZ, Invitrogen) or the chimeric construct (pU1/FIB) by long-term selection for zeomycin resistance. Human osteosarcoma (MG63) cells (Sakai, et al., *J. Cell Biol.*, 103; 2499, 1986) were grown to 60% confluence and 25 transfected with either linearized pU1/FIB or a lac Z reporter gene construct (pZeoSVLacZ, Invitrogen), used as a control. The transfections were performed using a DOTAP liposome formulation (Boehringer Mannheim, 1 mg/ml) according to manufacturer's instructions. Cells were grown in MEM media (Cellgro) with 10% FCS

and 250 µg/ml of zeocin (Invitrogen), and were maintained in selection for 14 days prior to the isolation of clonal colonies. Mono- and polyclonal colonies were established and grown to confluence.

Figure 3 shows an immunohistochemical analysis of MG63 cells that were stably transfected with an expression construct (pZeoSVLacZ, Invitrogen) lacking targeting sequence (panels A and C) or pU1/FIB (panels B and D). The antibodies either recognized epitopes in fibrillin-1 (A and B) or fibronectin (C and D). Analysis was performed in duplicate for each of three independent clonal colonies for each construct, with identical results. Representative fields are shown at 63X magnification.

Untransfected MG63 cells showed a pattern of protein deposition that was indistinguishable from that for cells transfected with pZeoSVLacZ.

Fibrillin-1 was strikingly absent upon immunohistochemical analysis (Eldadah et al., *J. Clin. Invest.*, **95**; 874, 1995) using either anti-fibrillin-1 mAb 69 (a gift from L. Y. Sakai) or an anti-fibronectin mAb (Sigma) of cells harboring pU1/FIB, while cells transfected with pZeoSVLacZ showed a pattern of protein deposition that was indistinguishable from untransfected controls (Figure 3). The lines harboring pU1/FIB could not be distinguished from mock-transfected or untransfected cells upon immunohistochemical analysis with a monoclonal antibody to fibronectin, suggesting specificity for the targeting process.

Antisense RNAs expressed by pU1/FIB might inhibit fibrillin-1 expression by multiple mechanisms. Ribozyme cleavage would remove the 5' cap from targeted transcripts, an event predicted to effect their rapid degradation. Alternatively, in the absence of ribozyme cleavage, involvement of the initiating AUG codon in duplex formation would impair translation.

The magnetic porous glass direct mRNA purification technique was used to isolate poly(A) RNA (according to the manufacturer's instructions, CPG, Inc.) and Northern blot analyses were performed. Electrophoresis of 3.5 µg of mRNA was performed under denaturing conditions, as previously described (Lehrach, et al.,

*Biochemistry*, 16; 4743, 1977; D.A. Goldberg, *Proc. Natl. Acad. Sci. USA*, 77; 5794, 1980). The gel was exposed to 60 mJoules UV light to facilitate transfer of large mRNA species. RNA was transferred to nylon membrane using the turboblotting system according to manufacturer's instructions (Schleicher & Schuell). The membrane was washed briefly in 2X SSC, crosslinked with 125 mJoules of UV light and prehybridized in Expresshyb (Clontech) for 1/2 hour at 68° C. Human cDNA probes encoding fibrillin-1 (nt 370-1183), β-actin (Clontech), and G3PDH (Clontech) were labeled by random priming (Feinberg, et al., *Anal. Biochem.*, 132; 6, 1983). The membrane was hybridized for 1 hour at 68° C, washed first in 2X SSC with 0.05% SDS at room temperature and then in 0.1X SSC with 0.1% SDS at 50° C.

Figure 4 shows a Northern blot analysis of 3.5 µg of poly(a) RNA extracted from confluent MG63 cells that were untransfected (lane 1), or stably transfected with either pZeoSVLacZ (Lane 2) or pU1/FIB (lane 3-5). Cells for lanes 1 and 3 were polyclonal, while cells for lanes 2, 4 and 5 were derived from monoclonal colonies. Hybridization was performed with radiolabeled cDNA probes complementary to fibrillin-1, β-actin, and G3PDH transcripts.

Northern blot analysis of multiple clonal colonies harboring pU1/FIB revealed undetectable levels of FBN1 message (Figure 4). FBN1 transcripts were easily detected upon Northern analysis of RNA extracted from untransfected and mock-transfected cells. All cell types showed comparable amounts of β-actin and G3PDH RNA, confirming the specificity of targeting.

A similar U1 construct was created containing U1 snRNA stemloop structures and an antisense core aimed at inhibiting rent-1 expression. Functional analysis of transfected cells showed a significant up-regulation of the steady-state abundance of transcripts derived from endogenous nonsense alleles.

## SUMMARY

These data suggest that most, if not all of the inhibitory effect was achieved at the level of target message abundance, presumably by reducing mRNA stability.

Although an influence upon transcriptional efficiency cannot be excluded, it is not intuitive given the site and nature of the predicted targeting sequence-target interaction.

Based upon previous studies, the antisense-induced mRNA degradation most likely takes place within the nucleus (Murray, et al., in *Modern Cell Biology*, J.A.H. Murray, Ed.

(Wiley/Liss, New York, 1992), pp 1-49 M.Cornelissen, *Nucleic Acids Res.*, 17; 7203, 1989.). Three elements of nuclear pre-U1 RNA, the 5' m7G cap, the 3' terminal stem

loop, and sequences in the 5'-terminal 124 nucleotides, contribute to the stability of pre-

U1 and snRNA and to efficient nuclear export (Neuman de Vegvar, et al., *Mol. Cell.*

*Biol.*, 10; 3365, 1990; Terns, et al., *Genes Dev.*, 7; 1898, 1993; Yuo, et al., *ibid.*, 3; 697,

1989; Izaurralde, et al., *Nature*, 376; 709, 1995). Nuclear targeting of cytoplasmic U1

snRNA is influenced by hypermethylation of the 5' cap structure and binding of at least

one common U snRNP protein, both dependent upon the integrity of the Sm protein

binding site which has been altered in pU1/FIB (Guthrie, et al., *Annu. Rev. Genet.*, 22;

387, 1988). A sequence at the 5' end of the insert (AAUUGG) is highly similar to the

consensus site [PuA(U)nGPu, Pu = A or G] for Sm protein binding (Jones, et al., *EMBO J.*, 9; 2555, 1990.). It has also been shown that Sm binding sites are highly tolerant of

mutations including internal nucleotide substitutions and deletions (Jones, et al., *supra*).

Stable transfectin of cultured cells with an expression vector containing the isolated FBN1/ribozyme core sequence of pU1/FIB but no U1 snRNA secondary structure, under the transcriptional control of the SV40 promoter, resulted in no discernible inhibitory effect on the expression of fibrillin-1 transcript or protein. The oligonucleotides described above containing the 24 highly conserved nucleotides of hammerhead ribozymes flanked by complementary sequence for 10 codons in the translation start site region of the FBN1 mRNA, were cloned into the SpeI and EcoRI restriction sites for the polylinker of pZeoSV (Invitrogen). Fetal fibroblasts were stably

transfected with this vector. Immunohistochemical analysis of these clones was performed by methods identical to those described herein). These results suggest that selected properties of U1 snRNA and/or its promoter are contributing to the extreme efficiency of inhibition seen with the use of the chimeric cRNA.

5        Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Dietz, Harry C.

(ii) TITLE OF INVENTION: Delivery Construct for Antisense Nucleic Acids and Methods of Use

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Fish & Richardson P.C.
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- (C) CITY: La Jolla
- (D) STATE: CA
- (E) COUNTRY: USA
- (F) ZIP: 92037

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/742,943
- (B) FILING DATE: October 31, 1996
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Haile, Lisa A.
- (B) REGISTRATION NUMBER: 38,347
- (C) REFERENCE/DOCKET NUMBER: 07265/098001

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 619/678-5070
- (B) TELEFAX: 619/678-5099

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTGGCGAT CTCCAGCACT GATGAGTCCG TGAGGACGAA ACGCCCTCGA CGCAT

55

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTAGATGCGT CGAGGGCGTT TCGTCCTCAC GGACTCATCA GTGCTGGAGA TCGCC

55

## **CLAIMS**

1. A nucleic acid construct for suppressing gene expression comprising:
  - a 5' stem loop structure;
  - an antisense nucleic acid; and
  - a 3' stem loop structure.
2. The nucleic acid construct of claim 1, wherein the stem loop structures are unmodified U snRNA structures.
3. The nucleic acid construct of claim 2, wherein the U snRNA is U1.
4. The nucleic acid construct of claim 1, further comprising a promoter.
5. The nucleic acid construct of claim 4, wherein the promoter is a U1 snRNA promoter.
6. The nucleic acid construct of claim 4, wherein the promoter is a constitutive promoter.
7. The nucleic acid construct of claim 4, wherein the promoter is an inducible promoter.
8. The nucleic acid construct of claim 1, further comprising a ribozyme nucleic acid.
9. The nucleic acid construct of claim 8, wherein the ribozyme nucleic acid is located between the 5' and 3' stem loop structures.
10. The nucleic acid construct of claim 8, wherein the ribozyme nucleic acid is a hammerhead-type ribozyme.
11. The nucleic acid construct of claim 8, wherein a consensus sequence for ribozyme cleavage in a target nucleic acid is 5'-GUC-3' or 5'-GUA-3'.

12. The nucleic acid construct of claim 1, wherein the antisense nucleic acid is selected from the group consisting of rent-1, HPV E6, HIV, hyaluronic acid synthase, and fibrillin.
13. A method for suppression of gene expression comprising administering to a cell a suppressive-effective amount of the nucleic acid construct of claim 1, whereby expression of the gene is suppressed.
14. The method of claim 13, wherein the administering is ex vivo.
15. The method of claim 13, further comprising administering a modified nucleic acid encoding a wild-type polypeptide corresponding to the gene product of the gene being suppressed, wherein the modified nucleic acid is resistant to ribozyme cleavage and/or antisense inhibition.

PCT/US2013/03760

**DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS**  
**AND METHODS OF USE**

**Abstract of the Disclosure**

A novel nucleic acid construct for delivery of antisense targeting sequences is  
5 provided. The construct includes intact stem loop structures and an antisense nucleic acid. Optionally, a ribozyme nucleic acid is included in the construct. The construct is useful for inhibition of selected genes in a cell. This allele-specific targeting is also useful in combination with replacement gene therapy.

19439.LJ1

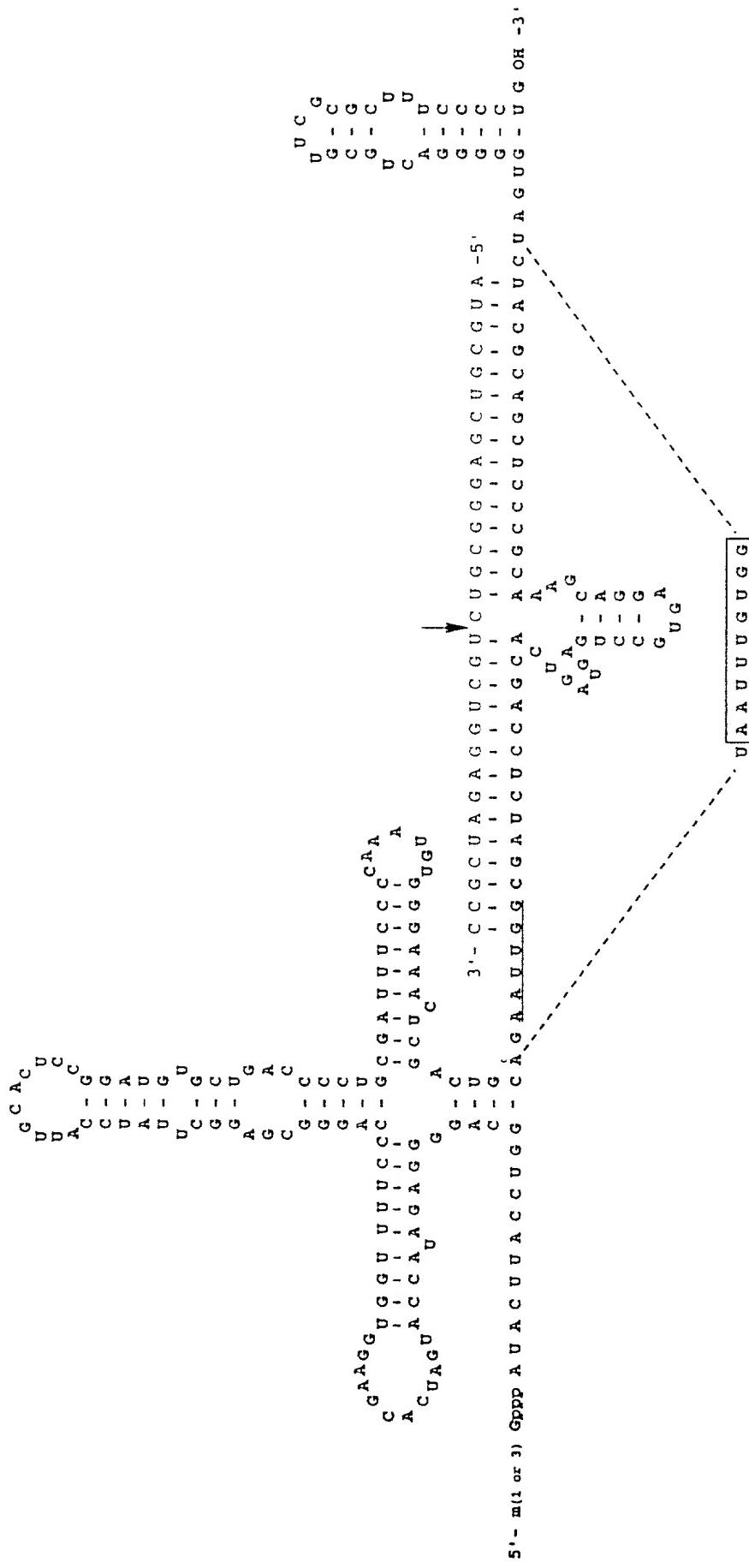


FIG. 1

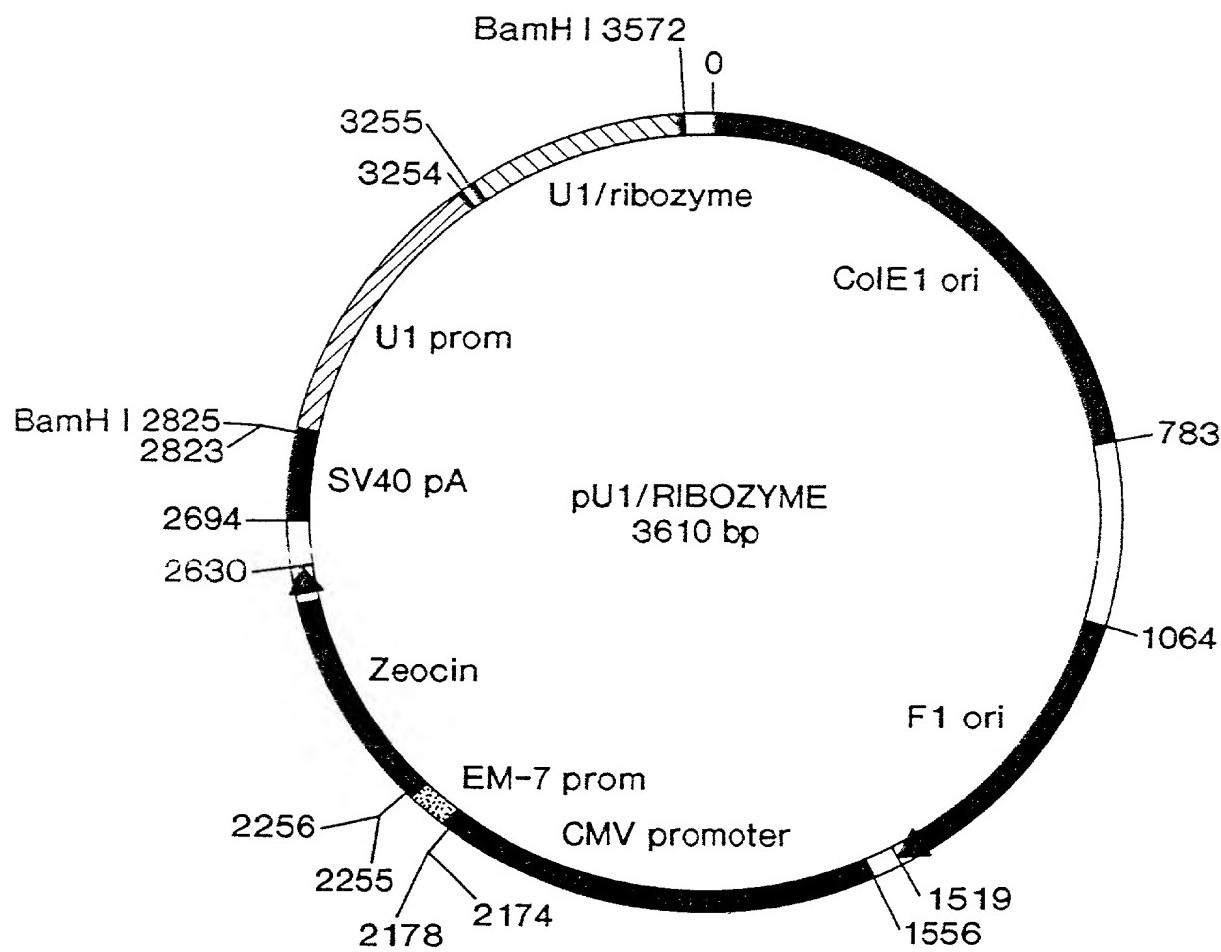


FIG. 2



FIG. 3A

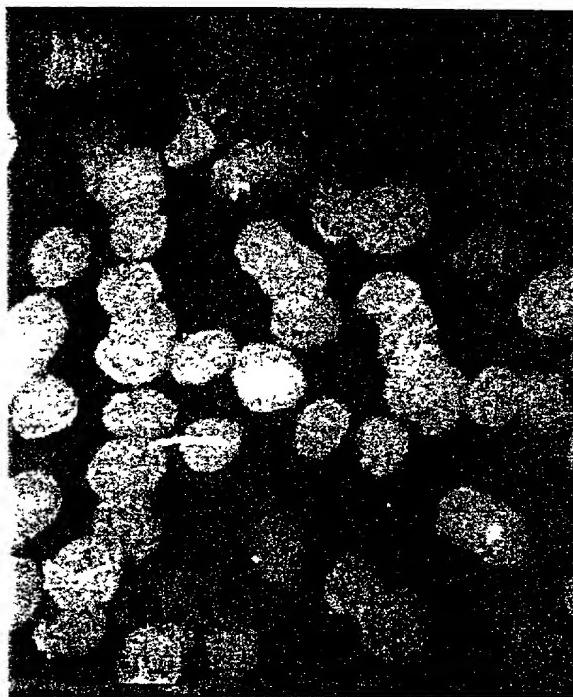


FIG. 3B

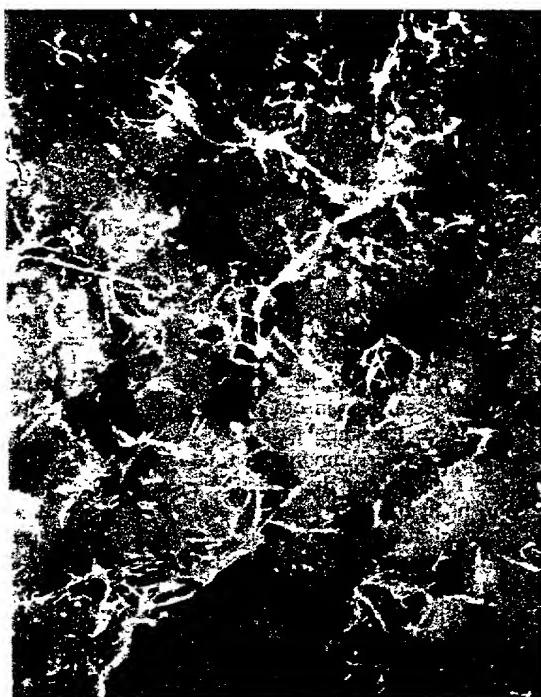


FIG. 3C

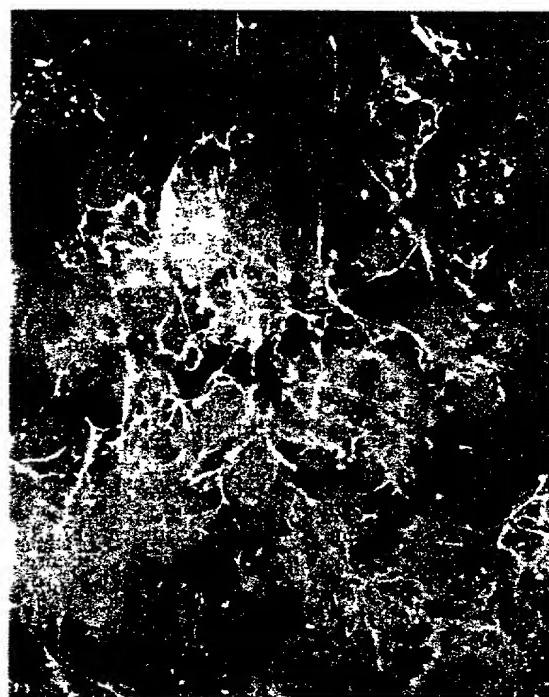


FIG. 3D

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1 2 3 4 5



Fibrillin-1



β-Actin

**FIG. 4**

PATENT

ATTORNEY DOCKET NO. 07265/098002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Harry C. Dietz  
Parent Serial No: 08/742,943 Art Unit:  
Parent Filed: October 31, 1996 Examiner:  
Serial No: Unknown  
Filed: September 29, 1998  
Title : **DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS AND  
METHODS OF USE**

Assistant Commissioner for Patents  
Washington, DC 20231

PERMISSION TO USE SEQUENCE LISTING

The above-identified patent application lacks a substitute paper copy of the Sequence Listing for inclusion into the Specification, as well as a computer readable form of the Sequence Listing. Applicants respectfully direct the attention of the Office to the following:

1. A complete paper copy of the Sequence Listing is to be inserted in the Specification starting at page 30 . This paper copy is identical to the paper copy of the Sequence Listing which is included in the Specification of the priority patent; to wit, U.S. Patent Application Serial No. 08/742,943(the "943 application"), filed on October 31, 1996.

"EXPRESS MAIL" Mailing Label Number EM153704389US  
Date of Deposit September 29, 1998  
I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

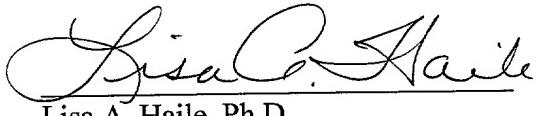
Duncan Clark

DUNCAN CLARK

2. A computer-readable form of the Sequence Listing identical to the paper copy of the Sequence Listing is on file in the Patent Application Serial No: 08/742,943 (the "943 application"), filed on October 31, 1996. Pursuant to 37 CFR §1.821(e) I, reference to the computer-readable form on file in the '943 application is hereby made, which computer-readable form was filed in the '943 application on September 16, 1997. Said computer-readable form is identical to both the paper copy of the Sequence Listing on file in the '943 application and the paper copy of the Sequence Listing submitted in the present application.

Applicants submit that the foregoing satisfies the requirements of Rule 1.821. If there are any questions regarding this request, the Office is invited to contact the undersigned.

Respectfully submitted,



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70175.LJ1

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(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTGGCGAT CTCCAGCACT GATGAGTCCG TGAGGACGAA ACGCCCTCGA CGCAT

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTAGATGCGT CGAGGGCGTT TCGTCCTCAC GGACTCATCA GTGCTGGAGA TCGCC

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COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled DELIVERY CONSTRUCT FOR ANTISENSE NUCLEIC ACIDS AND METHODS OF USE, the specification of which

- is attached hereto.  
 was filed on October 31, 1996 as Application Serial No. \_\_\_\_\_ and was amended on \_\_\_\_\_.  
 was described and claimed in PCT International Application No. \_\_\_\_\_ filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Lisa A. Haile, Ph.D., Reg. No. 38,347; June M. Learn, Reg. No. 31,238, John R. Wetherell, Jr., Reg. No. 31,678, and Barry E. Bretschneider, Reg. No. 28,055.

Address all telephone calls to Lisa A. Haile, Ph.D. at telephone number 619/678-5070.

Address all correspondence to Lisa A. Haile, Ph.D., Fish & Richardson P.C., 4225 Executive Square, Suite 1400, La Jolla, CA 92037.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: Harry C Dietz

Inventor's Signature: H. Dietz Date: 12/2/96

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Citizen of: United States

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